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Kininogen expression by rat vascular smooth muscle cells: Stimulation by lipopolysaccharide and angiotensin II

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Abstract

To identify the presence of a local kallikrein–kinin system in vascular wall, we have studied whether rat vascular smooth muscle cells (VSMC) express kininogen in vitro and in vivo. Western blots using anti-T-kininogen antibody revealed the presence of T-kininogen in conditioned medium of cultured VSMC. T-Kininogen secretion by VSMC was markedly enhanced by the addition of lipopolysaccharide (LPS), angiotensin II (AII) and phorbol 12-myristate 13-acetate (PMA) to the culture. Experiments using specific inhibitors for protein kinases and on the PMA-induced down-regulation of protein kinase C suggested that a protein kinase C-dependent or unidentified pathway is involved in AII or LPS action, respectively. The intravenous injection of LPS (0.5 mg/kg) resulted in an increase in T-kininogen mRNA levels in the vascular smooth muscle of rat aorta, peaking at 16 h. Polyacrylamide gel electrophoresis of cDNA products generated by reverse transcription–polymerase chain reaction (RT–PCR) from aortic mRNA using primers specific for either T- or low-molecular-weight kininogen revealed that rat vascular smooth muscle expressed T-kininogen gene but not low-molecular-weight kininogen gene, and that LPS exclusively stimulated T-kininogen expression. The mRNA for high-molecular-weight kininogen was undetectable in either aortic smooth muscle or cultured VSMC by means of RT–PCR analysis. RT–PCR using specific primers for rat tissue kallikrein genes showed that aortic smooth muscle expressed *KLK1* (true kallikrein) mRNA, but not *KLK10* (T-kininogenase) mRNA. These results demonstrated that rat VSMC are a source of T-kininogen but not of low-molecular-weight- or high-molecular-weight kininogen, in contrast to the expression of true kallikrein but not of T-kininogenase by these cells. 0167-4889/98/$ – see front matter

Keywords: Kininogen; Kallikrein; Vascular smooth muscle cell; Lipopolysaccharide; Angiotensin II; (Rat)

1. Introduction

The vascular tissues have been recognized as an endocrine and paracrine organ with a variety of functions involved in the regulation of blood pressure. The kallikrein–kinin system is one of the endogenous vasodepressor systems regulating blood pressure and generates potent vasodilating peptides, kinins, by a proteolytic activity of kallikreins on spe-

Abbreviations: VSMC, vascular smooth muscle cells; LMW-kininogen, low-molecular-weight kininogen; HMW-kininogen, high-molecular-weight kininogen; RT–PCR, reverse transcription–polymerase chain reaction; LPS, lipopolysaccharide; AII, angiotensin II; Bt_2cAMP, dibutyryl cyclic AMP; PMA, phorbol 12-myristate 13-acetate; IL-1, interleukin-1; IL-6, interleukin-6; TNF, tumor necrosis factor; HBSS, Hanks' buffered salt solution; DME, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum

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cific substrates kininogens [1]. The existence of an endogenous kallikrein–kinin system within the vascular wall [2] is supported by the following evidence: kallikrein is present in and is released from rat vascular tissue [3] or vascular smooth muscle cells (VSMC) in culture [4], rat VSMC contain mRNA for glandular kallikrein [5], and rat VSMC secrete a kininogen-like protein which releases a kinin on trypsin digestion [4]. This evidence suggests that both glandular kallikrein and kininogen are supplied locally within the vascular wall. However, no further characterization of the kallikrein–kinin system in vascular tissues has been reported.

The purpose of the present study was to clarify which species of kininogens is expressed by rat VSMC and what kinds of factors regulate the expression. Our results showed that a major kininogen synthesized by rat VSMC was T-kininogen, and that the synthesis was stimulated by lipopolysaccharide (LPS) and angiotensin II (AII).

2. Materials and methods

2.1. Materials

The following chemicals were obtained from commercial sources: LPS (*Staphylococcus typhosa* 0901) from Difco (Detroit, MI, USA); AII and bradykinin from Peptide Institute (Osaka, Japan); dibutyryl cyclic AMP (Bt_2cAMP) from Boehringer-Mannheim-Yamanouchi (Tokyo, Japan); phorbol 12-myristate 13-acetate (PMA) from Nacalai tasque (Kyoto, Japan); Immobilon-PSQ membrane from Millipore; recombinant mouse interleukin-6 (IL-6; 1×10^8 U/mg) from Genzyme; H-7, H-89 and W-7 from Funakoshi (Tokyo); and monoclonal anti-α-smooth muscle actin–alkaline phosphatase conjugate from Sigma. Recombinant human interleukin-1α (IL-1; 2×10^7 U/mg) and tumor necrosis factor-α (TNF; 3×10^6 U/mg) were donated by Dainippon Pharmaceutical (Osaka, Japan).

2.2. Isolation of smooth muscle and VSMC from rat aorta

Sprague–Dawley male rats, weighing 140–160 g, were anesthetized with ether and decapitated. Thoratic aorta (about 5 cm) were removed, and placed in a petri dish containing ice-cold Hanks' buffered salt solution (HBSS). The aorta was rinsed several times with HBSS, stripped of adventitia and denuded of endothelium with forceps. VSMC were grown from the explants of aorta without endothelium and were cultured in Dulbecco's modified Eagle's medium (DME) containing 15% fetal bovine serum (FBS). They were passaged every 10 days by harvesting with trypsin/EDTA and seeding at a 1:5 ratio in 28-cm^2 dishes. For experiments, cells after three to six passages were seeded into 75-cm^2 dishes or 24-well culture plates, fed every other day, and used at confluence (5–10 days). More than 95% of cells were positively stained with monoclonal anti-α-smooth muscle actin–alkaline phosphatase conjugate. Established smooth muscle cell line A7r5 cells, originating from the embryonic thoracic aorta of a DB1X rat, were obtained from Dainippon Pharmaceutical (Osaka, Japan) and maintained in DME containing 15% FBS.

2.3. Western blots of T-kininogen

VSMC in a 75-cm^2 dish at confluence were washed three times with FBS-free DME, then cultured in FBS-free DME for 3 days. Conditioned medium was harvested, dialyzed thoroughly against distilled water at 4°C, then lyophilized. The lyophilized sample was resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis in a 10% gel according to the method of Laemmli [6]. The resolved proteins were electrophoretically transferred to a nitrocellulose membrane, which was then blocked with 10% skimmed milk before being incubated with rabbit anti-rat T-kininogen antibody. Rabbit IgG on the membrane was detected by the avidin–biotin–peroxidase complex method according to the instruction manual issued by Bio-Rad Laboratories.

2.4. Detection of kininogen mRNAs in smooth muscle or VSMC of rat aorta

Expressions of kininogen mRNAs in smooth muscle or VSMC of rat aorta were determined by reverse transcription–polymerase chain reaction (RT–PCR) followed by Southern blotting using T-kininogen cDNA or high-molecular-weight (HMW) kininogen cDNA as a probe, as follows.

Total RNA was extracted with acid guanidinium–phenol–chloroform [7] from isolated smooth muscle or cultured VSMC. Total RNA (1 μg) was reverse transcribed in a 20 μl reaction mixture containing 50 pmol of the reverse primer, 2 μl of 10 mM dNTP, 2 μl of 10×PCR buffer (0.1 M Tris–HCl (pH 8.3), 500 mM KCl), 4 μl of 25 mM $MgCl_2$, 1 unit of RNase inhibitor, and 1 unit of cloned Moloney murine leukemia virus reverse transcriptase (GeneAmp RNA PCR kit; Takara, Japan). The RT reaction mixture was incubated at 42°C for 15 min, 99°C for 5 min, then 5°C for 5 min to allow synthesis of the first strand of cDNA. The cDNA was amplified in a 100 μl reaction mixture containing 50 pmol of the forward primer, 8 μl of 10×PCR buffer, 4 μl of 25 mM $MgCl_2$, and 2.5 units of *Taq* DNA polymerase. Eighteen to 25 cycles proceeded as follows: denaturation at 95°C for 1 min, annealing at 53°C for 2 min and extension at 72°C for 3 min. RT–PCR products (5 μl) were Southern blotted and autoradiographed using a Fuji Film Bio Imaging Analyzer BAS1000 (Fuji Film, Tokyo). We designed a set of primers common to both T- and low-molecular-weight (LMW) kininogen cDNAs, because the structures of these kininogen cDNAs are highly homologous [8]. The forward and reverse primers for both T- and LMW-kininogen cDNAs were 5′-ACATCACAGGTGGTTGCTGGA-3′ (nucleotides 961–981) and 5′-AGTACAAGGGCAGACTCTCA-3′ (nucleotides 1256–1275), respectively. The forward and reverse primers for HMW-kininogen were 5′-TACATGAGACCTTGGGAGAA-3′ (nucleotides 1105–1124) and 5′-AGTCCTGAAAACCACGAGAG-3′ (nucleotides 1701–1720), respectively. The probe for Southern hybridization of T-kininogen or HMW-kininogen was a 702-bp *Sau*3A1 fragment of T-kininogen cDNA [8] or a 616-bp fragment of HMW-kininogen cDNA, respectively.

2.5. Separation and detection of T- and LMW-kininogen mRNAs

To analyze RT–PCR products of T- and LMW-kininogen mRNAs, we used a fluorophore Cy5-labeled 5′-primer (Pharmacia) for RT–PCR, then the amplified cDNA products were separated and detected using an ALFred DNA sequencer (Pharmacia) as follows. RT–PCR was carried out with a Cy5-labeled 5′-primer as described above, then the product (1 μl) was mixed with 6 μl distilled water and 5 μl formamide, and heated at 95°C for 5 min. A sample (1 μl) was then electrophoresed on a denatured polyacrylamide gel containing 5% acrylamide and 42% urea using a DNA sequencer. Elutions of Cy5-labeled cDNA products were monitored by a fluorodetector of the sequencer. The retention times for T- and LMW-kininogen cDNA products were determined by the electrophoretic profile of RT–PCR products from authentic T- or LMW-kininogen cRNA. cRNAs were prepared using SP6/T7 Transcription Kit (Boehringer-Mannheim, Tokyo) from respective cDNAs that had been cloned from RT–PCR products of rat liver using TA Cloning Kit (Invitrogen, CA, USA).

2.6. Detection of tissue kallikreins KLK1 and KLK10 mRNAs in smooth muscle or VSMC of rat aorta

Expressions of *KLK1* (true kallikrein) and *KLK10* (T-kininogenase) mRNAs in aorta muscle or VSMC were determined by RT–PCR followed by Southern blotting, using specific primers and probes as described by McDonald et al. [9].

2.7. T-Kininogen radioimmunoassay

T-Kininogen was measured by radioimmunoassay using ^{125}I-labeled T-kininogen and rabbit anti-T-kininogen antibody, as described previously [10].

2.8. Statistical analysis

Results are presented as means ± S.D. The statistical significance of the difference between means was determined by Student's *t*-test.

3. Results

3.1. Identification of T-kininogen in conditioned medium of cultured VSMC

It is well documented that rat plasma contains three species of kininogens, HMW-, LMW- and T-kininogens, of which T-kininogen is a major kininogen produced by various tissues [11,12]. Thus we first

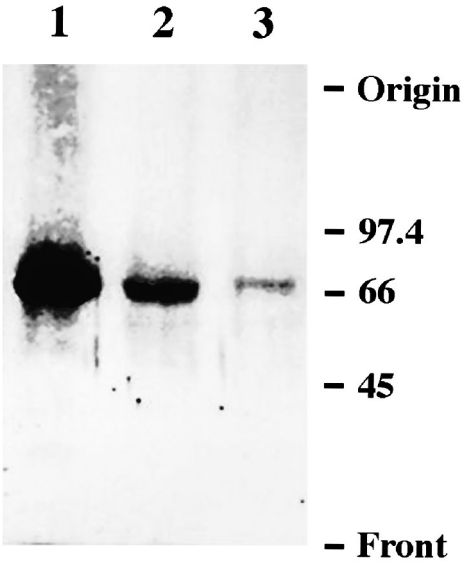

Fig. 1. Western blots of T-kininogen-like immunoreactive material in the conditioned medium of rat vascular smooth muscle cells (VSMC). Rat VSMC were cultured for 3 days, then the conditioned medium was harvested, dialyzed against distilled water and lyophilized. The rat T-kininogen (lane 1), rat serum (2 μl; lane 2) and lyophilized sample (lane 3) were resolved by sodium docecyl sulfate–polyacrylamide gel electrophoresis in a 10% gel and transferred to nitrocellulose membrane. The blot was incubated with anti-T-kininogen antibody followed by goat biotinylated anti-rabbit IgG, then with horseradish-labeled avidin. The numbers to the right of the blot represent molecular mass standards in kDa.

investigated whether the culture medium of rat VSMC contained a material that was reactive with a rabbit anti-T-kininogen antibody. Western blotting demonstrated that the conditioned medium contained a single immunoreactive band that was indistinguishable from rat plasma T-kininogen corresponding to a molecular mass of 68 kDa (Fig. 1). When Western blotting was carried out using a preimmune rabbit serum, neither the conditioned medium of VSMC nor purified T-kininogen revealed any immunoreactive bands (data not shown). T-Kininogen-like immunoreactivity was detected in the conditioned medium after 72 h culture at a level of 19.2 ± 13.2 ng/mg cell protein ($n = 4$) by radioimmunoassay.

3.2. *Effect of various agents on T-kininogen secretion by VSMC*

We recently reported that T-kininogen synthesis was enhanced by Bt_2cAMP and cytokines, such as IL-1 and TNF in rat fibroblasts [13]. To determine which factors regulate T-kininogen synthesis by VSMC, cells were cultured for 72 h with Bt_2cAMP, AII, LPS, PMA, or cytokines, such as TNF, IL-1, IL-6. T-Kininogen secretion by VSMC was markedly stimulated by AII (1 μM), LPS (5 μg/ml) and PMA (10 nM), while not affected by Bt_2cAMP (1 mM) and cytokines (500 U/ml) (Fig. 2). Lower concentrations of AII (10 nM) and LPS (0.5 μg/ml) were also effective in stimulating T-kininogen secretion (data not shown). Pretreatment of the cells with 0.1 μM PMA for 24 h abolished the stimulatory effect of AII, LPS and PMA on T-kininogen secretion (Fig. 3). To examine the role of protein kinases in AII- or LPS-induced T-kininogen response, cells were preincubated with protein kinase inhibitors H-7 (30 μM), H-89 (5 μM) or W-7 (70 μM) for 1 h. As shown in Fig. 4, AII-induced enhancement of the VSMC T-kininogen response was suppressed by H-7, but not by H-89 and W-7, while the effect of LPS was inhibited by these inhibitors to a similar extent.

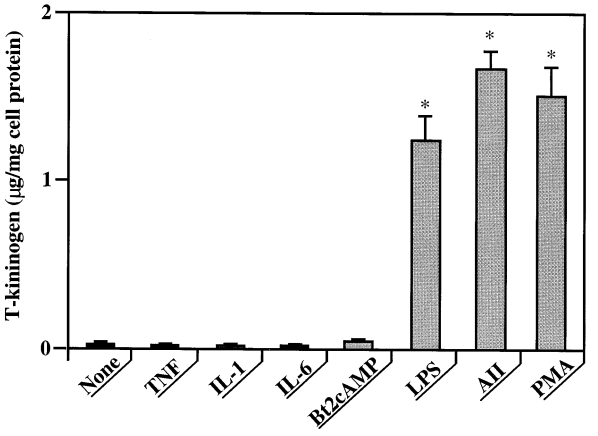

Fig. 2. The effects of various agents on the secretion of T-kininogen by rat VSMC. Rat VSMC were cultured in 24-well plates with or without various agents for 3 days, and the T-kininogen in the medium was assayed by T-kininogen radioimmunoassay. The results are expressed as the means ± S.D. of four wells. Untreated cells secreted 19.2 ± 13.2 ng/mg cell protein for 3 days' culture. Agents: tumor necrosis factor-α (TNF; 500 U/ml); interleukin-1α (IL-1; 500 U/ml); interleukin-6 (IL-6; 500 U/ml); dibutyryl cyclic AMP (Bt_2cAMP; 1 mM); lipopolysaccharide (LPS; 5 μg/ml); angiotensin II (AII; 1 μM); 12-myristate 13-acetate (PMA; 10 nM). Statistical analysis in comparison to unstimulated culture: $^*P < 0.001$.

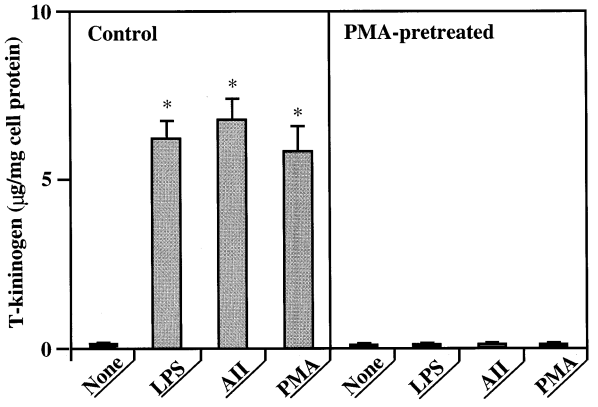

Fig. 3. Effects of PMA pretreatment on the LPS-, AII- or PMA-induced secretion of T-kininogen by rat VSMC. Rat VSMC were cultured for 1 day with 0.1 μM PMA. Cells were washed three times with fresh medium, then cultured with LPS (5 μg/ml), AII (1 μM) or PMA (10 nM) for 3 days, and T-kininogen in the medium was assayed by means of a T-kininogen radioimmunoassay. The results are expressed as the means ± S.D. of four wells. Statistical analysis in comparison to unstimulated culture: $*P < 0.001$.

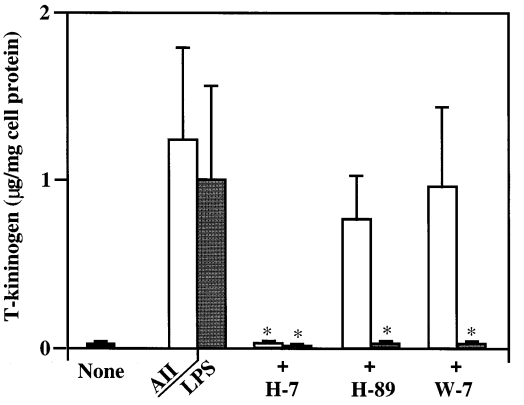

Fig. 4. Effects of protein kinase inhibitors on the AII- or LPS-induced secretion of T-kininogen by rat VSMC. Rat VSMC were incubated with or without protein kinase inhibitors H-7 (30 μM), H-89 (5 μM) or W-7 (70 μM) for 1 h, then cultured for 3 days following the addition of AII (1 μM) or LPS (5 μg/ml). T-Kininogen in the medium was assayed by means of a T-kininogen radioimmunoassay. The results are expressed as the means ± S.D. of four wells. Statistical analysis in comparison with the culture with AII alone or LPS alone, respectively: $*P < 0.001$.

3.3. Expression of T-kininogen mRNA in rat VSMC

Expression of T-kininogen mRNA in VSMC in vitro and in vivo was determined by RT–PCR followed by Southern blotting using T-kininogen cDNA as a probe. As shown in the representative autoradiogram in Fig. 5, a signal corresponding to the size of T-kininogen cDNA was faint in non-treated VSMC in vitro. However, the signal became intense

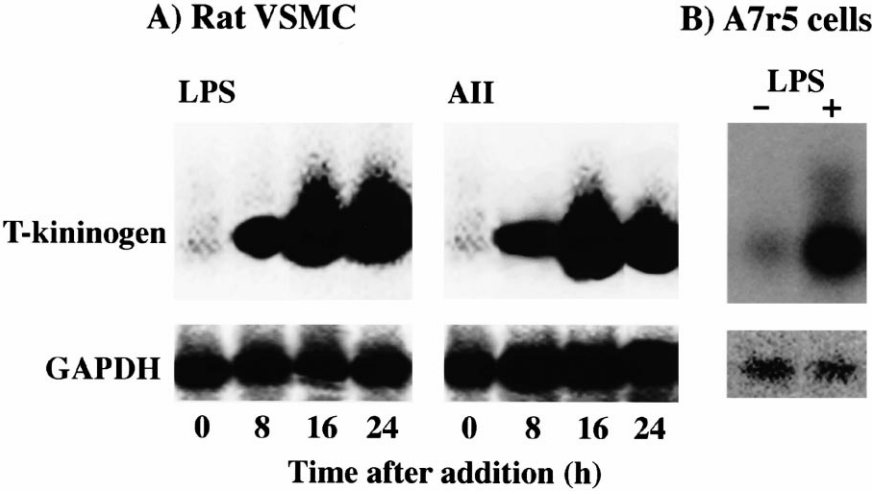

Fig. 5. Southern blot analysis of cDNA products from cultured rat VSMC (A) and A7r5 cells (B) generated by reverse transcription–polymerase chain reaction (RT–PCR) using primers and probe specific for T-kininogen. (A) Rat VSMC were cultured with LPS (0.5 μg/ml) or AII (1 μM) for several hours. (B) A7r5 cells were cultured with or without LPS (0.5 μg/ml) for 24 h. RT–PCR (25 cycles) for T-kininogen mRNA was performed with total RNA (1 μg) extracted from cells, then Southern blotting of the cDNA products was carried out using T-kininogen cDNA as a probe. The bottom panel shows Southern blots of the RT–PCR products amplified (20 cycles) by specific primers and probe for glyceraldehyde phosphate dehydrogenase (GAPDH) cDNA. Experiments were carried out four times for each treatment, and representative results were shown.

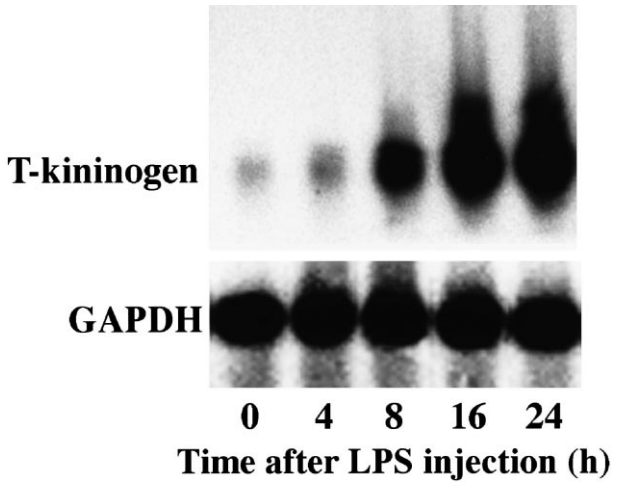

Fig. 6. Southern blot analysis of cDNA products generated from rat aortic smooth muscle by RT–PCR using primers and probe specific for T-kininogen. Rats were injected with LPS (0.5 mg/kg, i.v.), then four rats each were killed at 4, 8, 16, and 24 h by bleeding under ether anesthesia. Four untreated animals were killed as a control group. Thoracic aorta was isolated and the endothelium was denuded. Total RNA of aortic smooth muscle was extracted, and 1 μg the sample was analyzed by RT–PCR followed by Southern blotting, as described in Fig. 5. Representative Southern blottings are shown.

in the cells that had been cultured with LPS (0.5 μg/ml) or AII (1 μM), and peaked at 16–24 h after the treatment. An aortic smooth muscle cell line (A7r5) also expressed T-kininogen mRNA, and the expression was enhanced by an addition of LPS (0.5 μg/ml) in the culture (Fig. 5).

A marked induction of T-kininogen mRNA was also found in rat aorta in vivo after an intravenous injection of LPS (0.5 mg/kg) (Fig. 6). However, neither the intravenous bolus injection of AII (100 ng/animal) nor the continuous infusion of AII (50 ng/min) for 24 h by the subcutaneously implanted osmotic minipump influenced the levels of T-kininogen mRNA in the aorta (data not shown).

3.4. Expression of LMW-kininogen mRNA in rat aorta

It is well documented that T-kininogen is highly homologous to LMW-kininogen [8]. Since the primers used in this study for detecting T-kininogen mRNA could also amplify LMW-kininogen cDNA [14], it was conceivable that the RT–PCR products of VSMC were a mixture of T- and LMW-kininogen cDNAs. To determine this possibility, a RT–PCR was carried out using a fluorescent Cy5-labeled 5′-primer for both T- and LMW-kininogen cDNAs, then separation and detection of labeled cDNA products were performed with an ALFred DNA sequencer (Pharmacia) on the basis of a 6 bp difference in cDNA products between T- (315 bp) and LMW- (321 bp) kininogens [14]. Fig. 7 shows a representative electrogram in which the retention time for authentic T-kininogen cDNA was shorter than that for LMW-kininogen cDNA. The RT–PCR products of aorta collected from the non-treated rats revealed a faint peak corresponding to T-kininogen cDNA, whereas there was no peak corresponding to LMW-kininogen cDNA. The samples from LPS-treated rats showed a clear large peak of T-kininogen cDNA, but no peak was found at the retention time of LMW-kininogen cDNA. Thus it is likely that rat VSMC do not express LMW-kininogen gene, and that LPS stimulates the expression of T-kininogen gene but not that of LMW-kininogen gene.

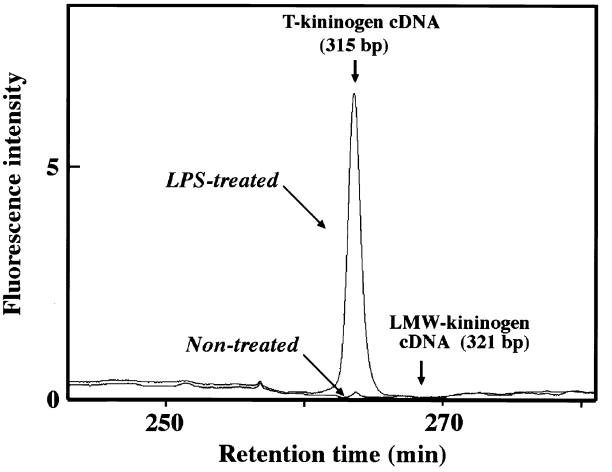

Fig. 7. Electrophoresis and detection of RT–PCR products of T- and LMW-kininogen mRNAs in the rat aortic smooth muscle by DNA sequencer. Total RNA samples were prepared from aortic smooth muscle of untreated or LPS-pretreated rats (24 h, 0.5 mg/kg, i.v.). The cDNAs transcribed from these samples were amplified by 18 cycles using a fluorescence-labeled 5′-primer and non-labeled 3′-primer which were specific for both T- and LMW-kininogen cDNAs. The labeled products were resolved by polyacrylamide gel electrophoresis and analyzed using an ALFred DNA sequencer (Pharmacia). The retention times for authentic T-kininogen and LMW-kininogen cDNAs were 263 and 269 min, respectively. Experiments were carried out with RNA samples from four non-treated and four LPS-treated animals, and representative results are shown.

3.5. Expression of HMW-kininogen mRNA in rat aorta

To determine if rat VSMC express HMW-kininogen, total RNA samples from aortic smooth muscle were examined by RT–PCR followed by Southern blotting using HMW-kininogen cDNA as a probe. We could not detect a signal corresponding to HMW-kininogen cDNA in the aortic muscle of either non-treated or LPS-treated rats nor in the cultured VSMC, in contrast to a distinct signal of HMW-kininogen cDNA in rat liver (data not shown).

3.6. Expression of tissue kallikrein mRNA in rat aorta

To determine whether rat aorta synthesizes T-kininogenase which is able to liberate T-kinin (Ile-Ser bradykinin) from T-kininogen [15], RNA samples from rat aortic smooth muscle were subjected to RT–PCR using specific primers for *rKLK10* (T-kininogenase) mRNA [9]. On Southern blotting, RT–PCR-amplified samples from rat submandibular gland RNA hybridized to the *rKLK10* probe, revealing an amplified 250-bp fragment, whereas no signal could be observed with RNA samples from aorta muscle and cultured VSMC (Fig. 8). No signal of *rKLK10* mRNA was also observed in RNA samples from aorta muscle of LPS-treated rats (0.5 mg/kg, i.v., 24 h) and those from VSMC that had been cultured with LPS (0.5 μg/ml) for 24 h (data not shown). In contrast, an amplified 347-bp fragment was observed by RT–PCR using primers specific for *rKLK1* (true kallikrein) with RNA samples from both aorta muscle and submandibular gland (Fig. 8).

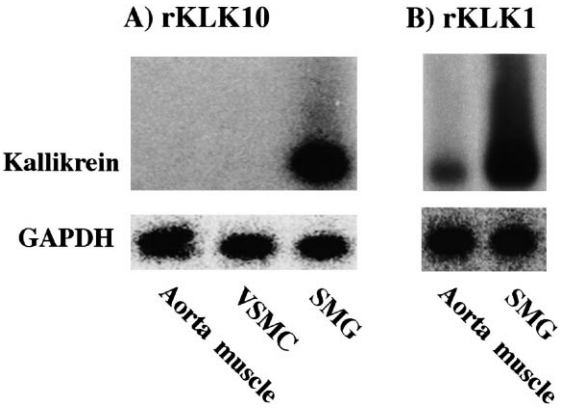

Fig. 8. Southern blot analysis of cDNA products generated from rat aortic smooth muscle or VSMC by RT–PCR using primers and probes specific for tissue kallikreins *rKLK10* (A) and *rKLK1* (B). RT–PCR (30 cycles) was performed with total RNA (1 μg) extracted from aorta muscle, VSMC or submandibular gland (SMG) using specific primers for *rKLK10* (A) or *rKLK1* (B), then Southern blotting of the cDNA products was carried out using oligonucleotide probe specific for each kallikrein. Experiments were carried out with RNA samples from four animals or cultures, and representative blottings are shown.

4. Discussion

Western blotting using anti-T-kininogen antibody showed that primary cultures of rat VSMC secreted T-kininogen-like protein in culture medium. However, since the structure of T-kininogen is highly homologous to that of rat LMW-kininogen [8], a possibility that the polyclonal antibody recognized LMW-kininogen as well as T-kininogen could not be excluded. Furthermore, primers for detecting T-kininogen mRNA by RT–PCR were specific for regions common to both T- and LMW-kininogen genes, indicating that the amplified fragments were a mixture of products derived from these genes. To determine whether rat VSMC express T- and/or LMW-kininogen, we measured relative amounts of RT–PCR products amplified from T- and LMW-kininogen mRNAs by separating and detecting these products on polyacrylamide gel electrophoresis [14]. We found a high level of T-kininogen cDNA in the RT–PCR products from the aorta muscle of LPS-treated rats, whereas there was an undetectable level of LMW-kininogen cDNA in the tissue of either non- or LPS-treated rats. We also attempted to detect HMW-kininogen mRNA in rat VSMC by means of the RT–PCR, but found no signal. Thus it is reasonable to conclude that rat VSMC express T-kininogen gene, but not LMW- and HMW-kininogen genes, and that LPS stimulates the expression of T-kininogen gene. Evidence that rat smooth muscle cell line A7r5 cells also expressed T-kininogen mRNA in response to LPS further supports this conclusion.

Addition of LPS to the cultured VSMC resulted in a marked elevation of T-kininogen mRNA in cells in

a similar time-course profile with the in vivo experiments, suggesting that a direct action of LPS on VSMC induces the expression of T-kininogen gene, probably via the LPS-receptor CD14 [16]. LPS was effective in vivo at the dose of 0.5 mg/kg i.v., which was comparable to the effective concentration of LPS at 0.5–5 μg/ml in vitro. Although addition of AII also produced a marked elevation of T-kininogen mRNA in cultured VSMC, we could not confirm this AII effect by the in vivo experiments: neither a bolus i.v. injection of AII (100 ng/animal), a dose sufficient to induce the elevation of blood pressure, nor a continuous infusion for 24 h at 50 ng/min per animal, which elevates plasma AII levels by three-fold, corresponding to about 50 pg/ml plasma [17], affected T-kininogen mRNA levels in the aorta muscle. The reason for the discrepancy between the in vitro and in vivo effects of AII seems to be due to much higher concentrations of AII in vitro (10–1000 nM) than in vivo. Thus it is doubtful whether the elevation of blood AII within the physiological range results in the expression of T-kininogen gene of rat vessels.

It is well documented that T-kininogen is one of the acute-phase proteins in the rat, whose synthesis in the liver increases after induction of inflammation [15,18], and IL-6 has been identified to be a mediator for this hepatic response [19,20]. However, as shown in this study, IL-6 was inactive to stimulate T-kininogen synthesis by rat VSMC. Other agents, such as dexamethasone, Bt_2cAMP, IL-1 and TNF, which have been reported to enhance the expression of T-kininogen gene in hepatocytes [21] or fibroblasts [13], also did not affect T-kininogen synthesis by VSMC. Like LPS and AII, PMA, a potent activator of protein kinase C, markedly enhanced the synthesis of T-kininogen by VSMC. Depletion of the PMA-sensitive protein kinase C by 24 h pretreatment of VSMC with PMA abolished not only the PMA effect on T-kininogen synthesis, but also the effect of AII or LPS, suggesting that the PMA-sensitive protein kinase C is involved in the enhanced expression of T-kininogen gene by these agents. In fact, the AII effect was clearly inhibited by a protein kinase C inhibitor H-7, but not by the protein kinase A inhibitor H-89 or calmodulin inhibitor W-7. However, in contrast to AII, the LPS effect on T-kininogen synthesis was uniformly inhibited by these inhibitors. Thus it is likely that AII stimulates the expression of T-kininogen gene by a protein kinase C-dependent pathway in rat VSMC, while the LPS effect may involve multiple protein kinases, one of which might be a PMA-sensitive protein kinase C. The fact that T-kininogen synthesis by VSMC is stimulated by PMA, but not by IL-6 and Bt_2cAMP, suggests that the regulation of T-kininogen expression is distinct from that in hepatocytes or fibroblasts, in terms of the responsiveness to these agents.

Rats have more than ten members of the tissue kallikrein family. Of these members, true kallikrein encoded by *rKLK1* has a kininogenase activity to liberate kinins from LMW- and HMW-kininogens [1], but not from T-kininogen [22], and T-kininogenase encoded by *rKLK10* liberates T-kinin from T-kininogen [23]. In the rat, vascular tissue contains a glandular kallikrein that is immunoreactive to antibody for rat urinary kallikrein [4] and has a kininogenase activity to release kinin from dog kininogen [3], indicating the presence of true kallikrein in rat vascular tissue. As expected, mRNA for true kallikrein was detectable in the aortic smooth muscle. However, as shown in this study, mRNA for T-kininogenase was not detectable in either aortic smooth muscle or cultured VSMC, in contrast to a distinct signal corresponding to T-kininogenase mRNA in submandibular gland [23]. Taken together, it appears that rat VSMC is a source of T-kininogen but not of T-kininogenase.

In conclusion, we have demonstrated that rat VSMC express T-kininogen gene but not LMW- and HMW-kininogen genes. We have also found that AII and LPS stimulate the expression of T-kininogen gene by VSMC, probably via a PMA-sensitive protein kinase C or multiple protein kinases including protein kinase C, respectively. Further studies will be needed to demonstrate the physiological role of T-kininogen in the vascular wall.

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